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Methodology Report

High-Resolution Whole-Mount *In Situ* Hybridization Using Quantum Dot Nanocrystals

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The photostability and narrow emission spectra of nanometer-scale semiconductor crystallites (QDs) make them desirable candidates for whole-mount fluorescent *in situ* hybridization to detect mRNA transcripts in morphologically preserved intact embryos. We describe a method for direct QD labeling of modified oligonucleotide probes through streptavidin-biotin and antibody-mediated interactions (anti-FITC and anti-digoxigenin). To overcome permeability issues and allow QD conjugate penetration, embryos were treated with proteinase K. The use of QDs dramatically increased sensitivity of whole-mount *in situ* hybridization (WISH) in comparison with organic fluorophores and enabled fluorescent detection of specific transcripts within cells without the use of enzymatic amplification. Therefore, this method offers significant advantages both in terms of sensitivity, as well as resolution. Specifically, the use of QDs alleviates issues of photostability and limited brightness plaguing organic fluorophores and allows fluorescent imaging of cleared embryos. It also offers new imaging possibilities, including intracellular localization of mRNAs, simultaneous multiple-transcript detection, and visualization of mRNA expression patterns in 3D.

1. Introduction

With the advent of cell type specific molecular markers, description and analysis of developmental morphogenesis has become possible in a number of biological systems. Gene-specific RNA probes are now extensively used as they enable visualization of gene expression patterns and thus provide valuable information regarding the role of specific genes during development, as well as regarding positioning and movement of a particular cell type at different stages of development. This technique, known as whole mount *in situ* hybridization (WISH), was initially performed using radioactive probes, but was radically simplified when non-radioactive probes were used successfully [2–4]. It is now widely used in several biological systems, including *Drosophila* [4], *Xenopus* [5], quail [6], *Dictyostelium* [7], and *Zebrafish* [8]. *In situ hybridization* (ISH) is a quite common technique among developmental research labs, yet its uses are quite diverse and include medical and prenatal diagnostics [9–16]. Not long after its introduction, protocols using two different labels for the RNA probes and two color substrates were described, allowing the detection of more than one transcript simultaneously [17]. However, a major limitation of these chromogenic multilabeling techniques is that the overlapping regions of expression are very difficult to discern. The use of fluorescent methods for detecting transcripts may overcome this limitation; yet fluorophores for single- as well as double-transcript visualization [18] in whole mounts have only been used extensively in the fly and to some extent in the zebrafish [8]. More recently, simultaneous fluorescent detection of three transcripts was reported in the chick, and multiplexing has been used to detect up to seven transcripts in the fly [19–21]. However, successful implementation of current fluorescent protocols is very rare, especially in the mouse or *Xenopus* embryos, and even then the fluorescent images are of insufficient quality and thus cannot rival the staining obtained with normal chromogenic substrates.

In addition, imaging the signal is complicated due to the strong autofluorescence of the embryos [22], which interferes with fluorescent detection of RNA and makes

the use of enzymatic amplification necessary in the *mouse*, *Xenopus, chick*, and other embryos. The amplification step is also necessary in the case of chromogenic protocols; however, this step undermines the resolving ability of both methods. Amplification reactions create a precipitate which is deposited in the area surrounding the RNA and then diffuses out. It therefore does not remain localized within the particular intracellular region or compartment where the RNA in question is localized. In fact, it often diffuses throughout the cell and sometimes even leaks outside of the cell being labeled [23]. This problem is less pronounced in the case of tyramide amplification, where the peroxidase reaction produces tyramide radicals that react covalently with proteins at the site of the reaction, reducing the diffusion radius appreciably [24, 25]. However, even in this case, the resolution is still limited by diffusion and depends on the time that the reaction is allowed to proceed [23]. To combat diffusion issues, direct labeling of the probe is required, as this allows single cell, as well as, intracellular resolution of the localization of a particular mRNA. In addition to improved resolution, direct labeling of the probe allows simultaneous hybridization of multiple mRNAs, as long as fluorophores can be spectrally resolved. It is also extremely simple and can be quantitative since the fluorescence of the probe can be calibrated [12]. Despite successful implementation of direct labeling of messenger RNAs in cell culture [26, 27], this has not been possible in embryos due to the low fluorescent intensities of organic fluorophores.

The use of fluorescent methods for detecting transcripts is highly advantageous compared to chromogenic methods, especially because it enables higher quality three-dimensional imaging, multiplexing different RNA species, and covisualization of RNA with proteins. As already mentioned, the major limitations preventing widespread use of fluorescence detection for WISH are the high endogenous background fluorescence of many embryos, as well as the limited brightness and photostability of organic fluorophores. A new type of inorganic fluorophore, namely, Quantum Dots (QDs) have been used recently in several systems *in vitro* for detection of proteins as well as *in vivo* for protein labeling and lineage tracing [28–42]. QDs were also used for *in situ* hybridization in clinical biopsies for the detection of multiple mRNAs with successful conjugation to oligonucleotide probes [43]. QDs have ideal optical properties for use in biology like strong fluorescent signal emission compared to organic and protein fluorophores [44, 45]. In addition, due to their longer excited state lifetime, their fluorescence can be separated from the background fluorescence with time-domain imaging [46]. Using QDs offers a number of other advantages over organic fluorophores including wide excitation spectra (which makes the use of a single excitation filter possible), narrow and tunable emission spectra (which reduces spectral overlap making the simultaneous use of more colors possible), large separation between the excitation and emission (which increases the detection sensitivity), and resistance to photobleaching [47, 48]. Their unique optical properties made QDs an ideal candidate for detecting multiple mRNAs in ISH protocols [49], and their high fluorescence intensity raised the possibility of using them for RNA detection in whole embryos. Previous efforts to do this met with aggregation issues and were time consuming since detection had to be done with RNA covalently linked to hydroxylated QDs [50]. Since then, we and others have reported new methods of creating hydrophilic QDs and hydrophilic QD conjugates have become commercially available [32, 38, 51]. Studies have also pointed out difficulties in using QDs for FISH experiments [52, 53], including steric hindrance [54], degradation of QD conjugates and adherence to tubes and tips [55].

In this work we explore the use of QDs in WISH experiments. We show that the greatest limitation of these nanocrystals is penetration, since commercially available QDs are quite large and therefore fail to penetrate the many cell layers of an embryo or are significantly trapped if they do. However, we have determined that proteinase K can render *Xenopus* embryos sufficiently permeable to allow QD penetration deep within embryonic tissues. More specifically, we produced fluorescein- (FITC-), biotin- , and digoxigenin (DIG) labeled RNA probes and used QD-antibody and QD-streptavidin conjugates to visualize them. Our experiments show that this is an extremely sensitive assay that significantly improved RNA detection sensitivity. We then employed QDs to visualize several RNA probes that had been used to perform WISH in *Xenopus* embryos. We demonstrate that QD detection of endogenous messenger RNAs is effective and that it can be used to provide WISH data of higher resolution than current techniques. Finally, we show that QDs can be used to carry out two-color *in situ* hybridization simultaneously. Therefore, the use of QDs to perform nonamplified fluorescent whole mount *in situs* in *Xenopus* embryos, one of the most highly autofluorescent (and thus demanding) vertebrate developmental organisms, suggests that QD whole-mount *in situs* will find successful applications in most developmental models.

2. Materials and Methods

2.1. Embryos. *Xenopus laevis* embryos from induced spawning [56] were staged according to Nieuwkoop and Faber [57]. Operation techniques and buffer (MMR) have been described [56]. *Xenopus* embryos were fertilized *in vitro* and dejellied using 2% cysteine-HCl, pH 7.8, then maintained in 0.1X Marc's Modified Ringer's (MMR).

2.2. Whole-Mount Immunofluorescence. *Xenopus laevis* embryos were fixed in 3.7% formaldehyde in MEMFA (2 hours at room temperature), and the vitelline envelope was manually removed with forceps. Permeabilization of embryos was carried out several ways: (1) overnight in 1X PBS supplemented with 0.5% Triton, and 1% DMSO (PBDT), (2) overnight in 1X PBS supplemented with 5% Triton and 1% DMSO, (3) two hours in 1X PBS supplemented with 0.2% SDS, and (4) for four hours in 1X PBS supplemented with 0.2% SDS or (5) for 25 minutes in 10^{-}g/mL Proteinase K. Embryos were then blocked for 2 hours in 1X PBS with 0.5% Triton, 5% BSA, and 1% Normal Goat serum. Primary antibody staining followed. Embryos were incubated with

biotin-conjugated phosphotyrosine (anti-4G10, Millipore) antibody overnight at 4°C at a dilution of 1 : 500 (in block solution). Embryos were then washed (3 × 10 min) in PBDT and incubated for 2 hours at room temperature with streptavidin conjugated Cy3 or QDs 655 nm at 1 : 500 dilution in fresh block solution. After incubation, embryos were washed in PBDT (3 × 10 min). For negative control experiments, embryos were blocked and then incubated with secondary conjugates only. Clearing of embryos was performed by immersion of the embryos in two parts benzyl benzoate and one part benzyl alcohol after methanol dehydration (Murray's Clearing Medium, BB : BA). The refractive index of BB : BA closely matches the refractive index of yolk thereby rendering *Xenopus* embryos nearly transparent. The embryos were imaged on a Zeiss Axio Imager Z1 using a Zeiss Axiocam MR3, the Axiovision software 4.7. Optical sectioning was achieved using a Zeiss Apotome structured illumination system.

2.3. In Vitro Transcription. Antisense digoxigenin - (DIG-), biotin- , and fluorescein - (FITC-) labeled Xbra (in CS2++), Edd, MyoD (in CS2++), Amylase (in pCR4Blunt-TOPO), Xa-1 (in pBSK+), cardiac actin (in pSP64), and LTBP1 (in CS2++) probes were synthesized by *in vitro* transcription from linearized plasmid using RNA polymerase SP6 or T3 and ribonucleotide mixture which results in RNA transcripts containing bio-UTP, FITC-UTP, or Dig-UTP (Roche). The manufacturer's protocol was followed with a modification in the total reaction volume which was scaled down to 20 μL. RNA probes from these reactions was purified using isopropanol/LiCl precipitation.

2.4. Chromogenic and Fluorescent Wholemount In Situ Hybridization. Biotin-, FITC-, and DIG-labeled RNA probes (transcribed as described above) were used to perform *in situ* hybridization using the protocol reported by Harland [58]. with some modification. Methanol was substituted with ethanol and 4% paraformaldehyde in PBS was used to fix the embryos instead of formaldehyde. After proteinase K treatment (5 min for chromogenic WISH and 25 minutes for QD-based WISH) embryos were blocked with 0.1% BSA, 10% sheep serum in 0.1% Tween in 1 X PBS(PBT) solution, and then washes were performed in PBS. After blocking, embryos were refixed for one hour in 4% paraformaldehyde followed by prehybridization at 65°C. For chromogenic WISH experiments, the original protocol was followed. However, for QD-based fluorescent WISH experiments the protocol was modified. After the last 0.2X SSC wash at 65°C the embryos were blocked with 1 X PBS + 0.1% BSA + 0.1% Tween for one hour and then transferred to a new vial which contained 1 mL of a 1: 500 dilution Qdot-streptavidin 705 nm, QD-anti-FITC or QD-anti-DIG 655 nm (Invitrogen) conjugates in blocking solution. The addition of 0.1% BSA and 0.1% Tween into the incubation buffer significantly improved penetration and decreased background without appreciably affecting the QD colloidal stability or the signal intensity. After the incubation, the embryos were washed in PBT (4 × 30 min) at room temperature. Embryos were then cleared in BB : BA (as described above). The fluorescent signal remains localized after clearing, and this allows data acquisition from different planes within the embryo without the need for sectioning. The embryos were imaged on an upright Zeiss fluorescent microscope with a Zeiss Axiocam and the Axiovision 4.7 software (using a custom filter set; excitation 300–460 nm, emission 500 nm longpass, dichroic 475 nm). When detecting weak signals, the Axiovision software allowed white balancing of the camera so that that the green background (in embryos viewed with a 420 long pass filter due to the bias of the autofluorescence towards shorter wavelengths) appears white, resulting in a major boost of the QD signal (605 peak emission), decrease of the threshold of detection and significantly better contrast. If the calibration of the camera is done properly, control embryos that are not labeled with QDs appear completely white under UV excitation without any traces of red. This color separation method has to be performed carefully and control embryos need to appear white otherwise the risk of generating false staining increases significantly. Due to the fact that the embryo has several distinct regions where the background fluorescence changes not only in terms of intensity but also in terms of spectral balance the region chosen for assignment of "white" was the region in which the background had the longest average wavelength. In this manner we ensured that long-average wavelength background regions would appear white, and shorter average wavelength regions would appear blue.

3. Results and Discussion

3.1. Proteinase K Facilitates QD Penetration in Xenopus Embryos. We first wanted to examine whether QDs could penetrate *Xenopus* embryos to a sufficient depth so as to allow specific deep tissue staining. We initially compared streptavidin-conjugated QDs to streptavidin-conjugated Cy3 in whole-mount immunostaining experiments for their ability to detect a biotinylated antibody against phosphotyrosine. This antibody was purposely selected due to its strong and specific staining pattern of cell-cell boundaries that allows easy visual confirmation upon successful labeling. Not surprisingly, the staining pattern obtained using QDs was very similar to the one obtained using Cy3 in the superficial cell layer of the embryo (upper area of Figure 1(a) showing superficial cells of the fin of a tadpole and data not shown). However, and in contrast to Cy3, QD staining could not be detected beyond the first cell layer indicating that QDs encountered penetration issues, most likely due to size restrictions (Figure 1(a) lower area showing the somites of the tadpole). Several approaches were employed to increase QD embryo permeability, including the use of harsh detergents like SDS, with limited success. However, use of proteinase K treatment enabled penetration of QDs into deep tissues and resulted in specific deep tissue staining (Figure 1(b) and Table 1). However, proteinase K treatment is not suitable for use with most whole-mount immunostaining antibodies as it may lead to degradation of the target antigen.

It should be noted that the best results were obtained using newly opened QD-streptavidin conjugates from Invitrogen. Unfortunately though, the performance of these

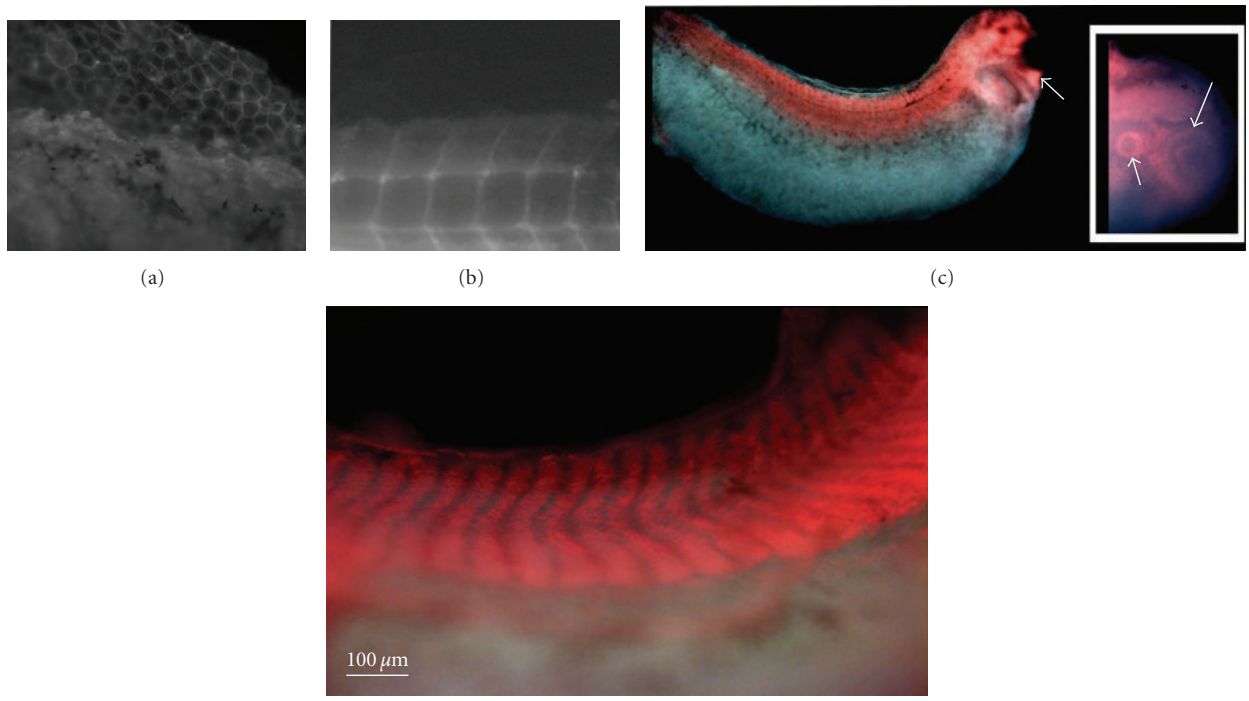

Figure 1: Proteinase K treatment is necessary to allow QD penetration and labeling of deep tissues in *Xenopus* and QD705 nm anti-Dig conjugates can be used for the detection of transcripts in wholemount *in situ* hybridization experiments. (a) Detection of biotinylated 4G10 (anti-Phosphotyrosine) antibody using streptavidin conjugated 655 nm QDs in a triton permeabilized embryo. Specific staining can be seen at the cell-cell boundaries of the ectodermal cells of the fin (upper part of the image) but not in the deep tissues (somites at the bottom part of the image). (b) Detection of biotinylated 4G10 (anti-Phosphotyrosine) antibody using streptavidin conjugated 655 nm QDs in a PK permeabilized embryo. Specific staining of the deep intersomitic boundaries can be seen. Superficial cells of the fin cannot be seen due to degradation of this delicate structure by the PK treatment. (c) QD705 nm anti-DIG antibody labeling of the probe for LTBP1 generates a staining pattern that closely matches the published expression for this mRNA [1]. QDs label the somites as well as anterior neural and neural crest tissues including the branchial arches and a region surrounding the eye. (d) Imaging of the QD labeling for LTBP1 in the somites at 10X magnification.

Table 1: Comparative chart of different permeabilization approaches used in whole mount immunostaining using a biotinylated anti-phosphotyrosine antibody (4G10 clone) and detected with Streptavidin conjugated QDs. Proteinase K treatment is the only approach which allowed deep tissue staining using QDs.

	Superficial Staining	Deep Tissue Staining
TRITON 1%	+++	–
TRITON 5%	+++	–
0.2% SDS 2 h	+++	+
0.2% SDS 4 h	+++	+
PK 25 min	+++	+++

conjugates was batch dependent and significantly diminished if QD-streptavidin conjugates were stored for more than two-three months, despite a six-month shelf life stated by the manufacturer. In addition, it was evident that use of QDs with emissions in the NIR (700 and 800 nm) provided a big improvement in detection sensitivity due to a significant reduction of tissue autofluorescence in this region of the spectrum. However, commercially available QDs with peak emissions in these wavelengths are quite large and suffer from even greater permeability problems. Consequently, there is an increased need for more reliable and smaller NIR QDs to become commercially available.

3.2. QD Labeling of LTBP1 in Xenopus Embryos. Given that proteinase K treatment facilitated QD penetration in *Xenopus* embryos and allowed specific deep tissue staining in whole-mount immunostaining experiments, we wanted to determine whether QDs could be used in WISH experiments in which proteinase K treatment is a standard permeabilization approach. We initially tested whether anti-digoxigenin-conjugated QDs could detect a DIG-labeled RNA probe specific for *Xenopus* Latent Transforming Growth Factor β Binding Protein 1 (LTBP1). As shown in Figure 1, anti-DIG QDs (see Figures 1(c) and 1(d)) gave a similar staining pattern of *Xenopus* LTBP1 to the published pattern obtained using the same probe but developed using a chromogenic reaction [1]. LTBP1 signal was detected in the head region including branchial arches and around the eye (shown as inset of Figure 1(c)) and the somites in agreement with the published expression pattern [1]. Furthermore, QD-labeling of LTBP1

(a) (b)

Figure 2: QD-streptavidin staining of LTBP1 compares favorably with the published staining achieved using standard chromogenic protocols in the deeper structures of the embryo [1]. (a) A transverse section from a whole mount *in situ* indicating the LTBP1 transcript expression pattern using QD705 nm streptavidin. The somitic staining obtained using QDs is identical to the published data using the chromogenic protocol [1], showing that the QD-streptavidin solution can penetrate and stain the deep areas of the somites. (b) A series of transverse optical sections of a QD-streptavidin-stained embryo for the LTBP1 message. The optical sections reveal that the staining previously identified as notochord by the chromogenic protocol is in fact somitic mesoderm flanking the notochord (nc: notochord, sm: somatic mesoderm).

exhibited high signal intensity and resolution confirming that a regular fluorescence microscope is sufficient for detection (see Figures 1(c) and 1(d). To further establish that the QD *in situ* protocol is capable of successful and specific staining, we sectioned and imaged whole-mount QD-stained embryos. As shown in Figure 2(a), QDs can penetrate deep into the embryo following proteinase K treatment and stain structures independently of their proximity to the free QDs in solution. Importantly, the use of QDs for *in situ* staining could result in more accurate determination of transcript expression patterns. Figure 2(b) shows a series of optical section images taken from a z-stack movie, using a fluorescence microscope, starting at the head region and moving posteriorly. As indicated, QD labeling survives the clearing protocol used to render *Xenopus* and other embryos transparent. More importantly, however, is that the optical sections demonstrate that the staining does not originate from the notochord as originally published [1], but rather from the somitic mesoderm flanking the notochord. Therefore, the additional spatial cues provided by optical sectioning can lead to a more accurate determination of an expression domain, further emphasizing the advantages of QD *in situs*.

3.3. QD Labeling of Specific Transcripts in Xenopus Embryos. The above results suggest that use of anti-DIG conjugated QDs in WISH can give highly specific staining of DIG labeled mRNA probes, even in deep tissues. To further determine the utility of this approach we tested whether QDs could also label alternative modified oligonucleotide probes through biotin-streptavidin and FITC- anti-FITC interactions, using a similar protocol. Figure 3(a) depicts *in situ* staining performed on a dissected *Xenopus* tadpole gut, against a biotin labeled amylase probe using either streptavidin-conjugated QDs or the chromogenic reaction. It is evident, from the images, that similar staining patterns are obtained; both the QDs and the chromogenic staining were restricted to the pancreas, where amylase RNA is expressed. Non-stained areas appear white due to background fluorescence that is present in all visible wavelengths. Despite high background, we obtained a good signal to noise ratio (sufficient to allow clear visualization and delineation of the expressing region) and excellent contrast in the most highly autofluorescent organ of the *Xenopus* tadpole.

We went on to test three well-known mRNAs, namely, (a) Endodermin (Edd: a pan-endodermal marker), (b) Xbra (an early mesodermal marker), and (c) MyoD (a gene encoding a DNA-binding protein that can activate muscle gene expression), which stain the gut, the mesodermal belt, and the muscle somites, respectively. As indicated in Figures 3(b) and 3(d), the use of QDs in *in situs* gives staining patterns that closely match the ones obtained using standard enzymatically amplified chromogenic reaction methods, while maintaining high resolution. The degree of resolution, however, varies with the transcript of interest and its respective expression pattern. For example, in the case of MyoD (Figure 3(b), FITC-labeled probe used), the posterior somites look fused when using the chromogenic protocol but are clearly distinct when using QDs. In contrast, QD staining of a biotin-labeled probe against Edd, which is expressed in the highly autofluorescent gut appears weak except at the anterior, where the gene is expressed at higher levels (Figure 3(c)). Alternatively, in the case of Xbra (Figure 3(d)), the staining of the chromogenic and the QD (against biotin-labeled probe against Xbra) *in situ* is almost identical. Hoechst was used to counter-stain the nuclei blue in this experiment, and the cleared embryo was visualized from the animal pole.

3.4. Simultaneous Labeling of Two Transcripts Using QDs. The fact that all three common modifications of RNA probes could be detected successfully with QDs raised the possibility that QDs could be used to detect two or more transcripts simultaneously. Achieving multiple transcript labeling using chromogenic protocols is a time consuming, stepwise process which, as explained earlier, results in the inability to distinguish areas of coexpression. In order to determine whether two transcripts can be visualized simultaneously, with the use

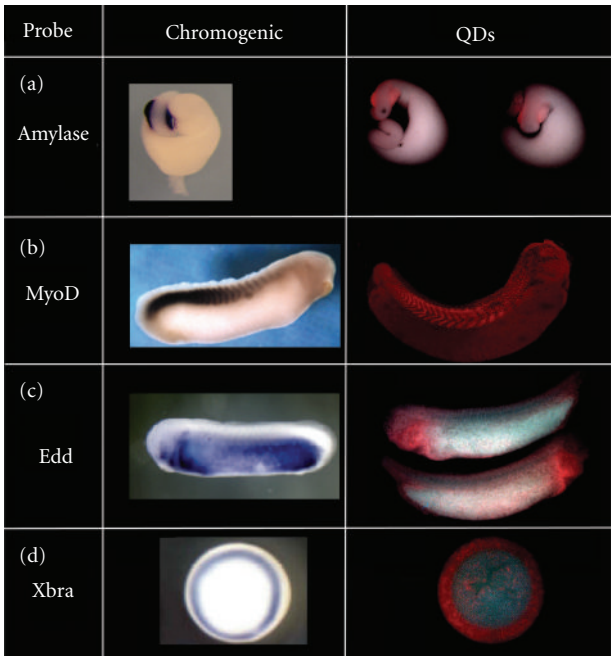

Figure 3: *In situ* hybridization using QDs compares favorably with chromogenic *in situ* hybridization staining for a number of well-characterized mRNAs. (a) 705 nmQD-streptavidin staining for amylase on dissected *Xenopus* guts, using a biotinylated amylase probe, is compared to the staining obtained by chromogenic reaction (left). The staining using QDs is identical to that using a chromogenic reaction and restricted to the pancreas, where amylase mRNA is expressed. It is worth noting that the pancreas, a morphologically identifiable organ, is extremely autofluorescent making detection of fluorescent staining difficult. (b) Comparison of the QD and chromogenic staining for MyoD a muscle marker (using a FITC-labeled probe). The 655 nmQD anti-FITC and the chromogenic staining are similar, but the QD staining gives much better resolution of the posterior somites. (c) Comparison of QD versus chromogenic staining for the Edd transcript, an endodermal marker expressed through the tadpoles gut at varying levels (using a Biotin-labeled probe). The staining using a chromogenic protocol is significantly stronger in this case and the 705 nmQD-Streptavidin seems restricted to the high expression regions. Careful observation reveals that the staining is present throughout the gut region but is masked by the intense autofluorescence of the gut. (d) Comparison of the QD staining versus the chromogenic staining for Xbra, a widely used mesodermal marker (using a Biotin-labeled probe). The marker is known to label the mesodermal belt at gastrula stages; both the chromogenic, as well as the QD-streptavidin protocols result in the same staining pattern consistent with the mesodermal belt.

of QDs, we generated two probes; a FITC-labeled probe against Xa-1 and a biotin-labeled probe against *Xenopus* cardiac actin. The two probes were hybridized at the same time and detected using spectrally distinguishable anti-FITC-conjugated and Streptavidin-conjugated QDs, respectively. As seen in Figures 4(a) and 4(b) both probes were visualized successfully demonstrating the ability of simultaneous detection of multiple transcripts using the QD *in situ* protocol.

In addition, due to the lack of enzymatic amplification, the resolving ability of this method is substantially better than that of amplified protocols. This is evident in Figures 4(c) and 4(d), where we present high-magnification images of whole-mount-stained embryos showing the intracellular mRNA localization for Xa-1 and LTBP1, respectively. As can be seen, QD labeling of transcripts is of extremely high resolution and enables distinction of intracellular localization patterns of mRNA. While LTBP1 localized in the cytoplasm, the Xa-1 transcript appeared to be concentrated near the plasma membrane at the cell-cell contact areas. Even though there is no evidence proving that the presented mRNA distribution coincides with the true intracellular localization of these transcripts, the fact that there are such dramatic differences in the signal patterns from different probes suggests that this is indeed the case. Future work will have to focus on closely examining the resolution of this method in model systems where direct, nonamplified *in situs* can be performed, using traditional fluorophores for comparison. Nevertheless, it is clear that this level of resolution cannot be achieved with existing methods for RNA transcript detection in *Xenopus,* which are mostly based on enzymatic amplification. We thus propose that use of the QD approach can simultaneously give macroscopic and intracellular data regarding the distribution of mRNAs in vertebrate embryos.

4. Conclusions

Herein we describe a new application of QDs in nonamplified whole-mount fluorescent *in situ* detection of endogenous mRNAs. The ideal optical properties of QDs provide unprecedented resolution and strong signal intensities that have not been possible to attain using traditional fluorophores. In fact, even though WISH has been available for more than a decade, the requirement for an enzymatic amplification step significantly limited the resolution of this method. Additionally, limitations of current fluorescent protocols have prevented widespread use of fluorescent *in situs* in most developmental models, with the exception of *Drosophila,* and to some extent zebrafish; direct visualization of highly abundant transcripts is possible in *Drosophila*, but not in most vertebrate models, in which an enzymatic amplification step is required. Fluorescent detection of a messenger RNA opens exciting possibilities in terms of imaging and can eliminate the need for sectioning samples. Optical sectioning provides better spatial cues and ensures correct identification of expression domains. It can also be used to create three-dimensional maps of expression, at a previously unattainable resolution, especially if the amplification step is eliminated. Intracellular localization of mRNA transcripts has only recently been investigated [59–63]. The study of mRNA localization is limited by the current *in situ* methods, both fluorescent and chromogenic, due to their relatively low resolving ability. Chromogenic reactions have been used successfully to localize mRNA transcripts in cultured cells but only for transcripts of very high abundance [60–63]. The method we describe is the first nonamplified fluorescent detection of mRNA *in situ* in *Xenopus*. Our protocol results in signal intensities sufficient for imaging on a regular epifluorescence microscope without the need

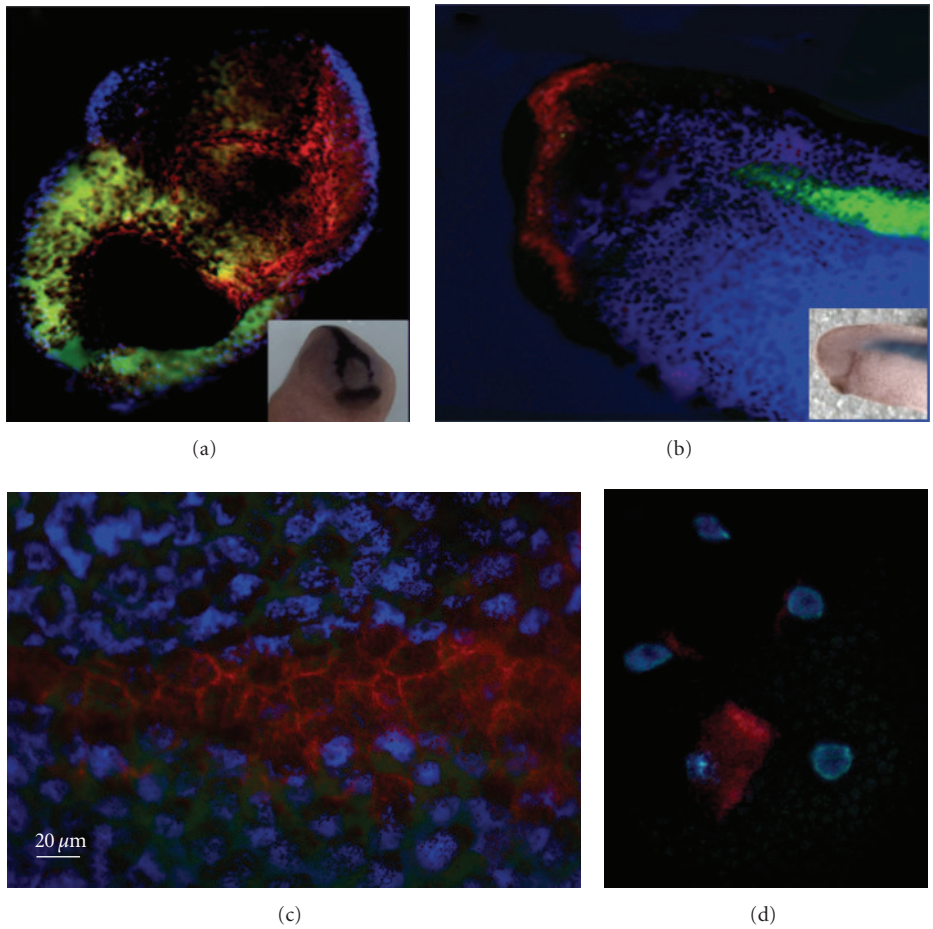

Figure 4: Double whole-mount *in situ* hybridization against cardiac actin and Xa-1 and the intracellular distribution of LTBP1 and Xa1. (a) A comparison of the staining pattern between the chromogenic and QD-based visualization of the FITC-labeled Xa-1 probe, shown in red, reveals that the QD staining is identical to that obtained using the standard chromogenic protocol. (b) An embryo processed using Biotin and FITC-labeled probes against cardiac actin (green) and Xa-1 (red), respectively. The two probes were visualized with spectrally resolvable QDs demonstrating that two color fluorescent *in situs* can be performed using QDs. The inset shows the chromogenic staining for cardiac actin for comparison. (c, d) Images of embryos processed for whole-mount *in situ* hybridization and counterstained with Hoechst (blue) at 20X (c) and 40X (d) magnification, showing differences in the intracellular distribution of the transcripts of Xa-1 (c) and LTBP1 (d), both shown in red.

for confocal microscopy. Of great significance, in terms of *Xenopus* and other opaque embryos like the chick, is the fact that the QD *in situ* staining is capable of remaining localized and fluorescent for more than an hour after the embryo is cleared. QD *in situs* also offer the potential for intracellular resolution of mRNA expression. This has not been possible using traditional chromogenic or fluorescent methods. In the case of *C. elegans,* where background is not a major issue, use of fluorescent antibodies to detect labeled RNA probes has to be carried out in conjunction with chromogenic amplified detection of the probes in order to get a comprehensive picture of the overall expression of a gene [64]. The amplified reaction detects low expressing regions and overall expression, whereas the fluorescent antibodies are used to resolve intracellular localization. The fact that our QD *in situ* protocol can do both in the highly autofluorescent *Xenopus* embryo, which is highly demanding, makes us confident that the implementation of QD *in situs* in other less demanding model systems will be met with equal or more success. More importantly, these results suggest that QDs could be introduced as alternative fluorophores in other fluorescent *in situ* hybridization assays where their spectral properties can offer significant advantages. Overall, our results demonstrate that QD *in situs* are a viable alternative to current ISH protocols, and they expand the uses of QDs in biology.

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